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Removal of the MDCK Cell Primary Cilium Abolishes Flow Sensing

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Abstract. The hypothesis that cell primary cilium is solely responsible for the flow-induced Ca^{2+} response in MDCK cells was tested by removal of the cilia from mature, responsive cells. Incubation of the cells with 4 mM chloral hydrate for 68 hours resulted in the complete loss of the primary cilia and in disorganization of microtubules, as visualized by immunofluorescence. When intracellular Ca^{2+} concentration was measured with Fluo-4, the elevation that normally accompanies an increase in fluid flow was abolished after 20 hours exposure to chloral hydrate. At this time, the primary cilia still remained attached to the cells but had become twisted and flexible. Twentyfour hours after return of the deciliated cells to normal medium, intracellular microtubule organization appeared normal, but primary cilia had not yet been expressed. The cells failed to increase intracellular Ca^{2+} in response to fluid flow until after they had been in normal medium for 120 hours, at which time the primary cilia were 3–4 μm long. Chloral hydrate did not impair the Ca^{2+} mobilization machinery, as the Ca^{2+} response to mechanical contact and the spread to neighboring cells was unaffected by the drug. We conclude that the primary cilium is the only sensor for the flow-induced Ca^{2+} response in MDCK cells and estimate that a single mechanically sensitive channel in the cilium could provide the requisite Ca^{2+} influx.

Key words: Chloral hydrate — Microtubules — Immunofluorescence — Calcium

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Introduction

In a previous study (Praetorius & Spring, 2001), we provided evidence that bending the primary cilium of MDCK cells with a micropipette results in a large increase in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, as a consequence of Ca^{2+} entry followed by Ca^{2+}-induced Ca^{2+} release (CICR) from the IP_3-sensitive Ca^{2+} stores. We further demonstrated that flowing the perfusate over the apical surface of the cells resulted in a similar increase by the same mechanism. The aim of the present study is to address whether the primary cilium is the only sensor responsible for the flow-induced Ca^{2+} response in MDCK cells. Although it was shown that pre-confluent cells, which lack primary cilia, did not respond to flow changes with an increase in $[Ca^{2+}]_i$ (Praetorius & Spring, 2001), their failure to respond could have been due to lack of production of the necessary mechanosensitive proteins. We, therefore, sought to develop a method by which the primary cilium could be removed from confluent cells that exhibited a flow response. We hypothesized that, if the primary cilium were the only sensing mechanism for fluid flow, mature cells lacking a cilium would become unresponsive and would only regain responsivity when the primary cilium grew back.

Primary cilia develop from the cell's mother centriole and are anchored to the basal body. It has previously been shown that long-term incubation with chloral hydrate removes cilia from *Paramecium caudatum* (Dunlap, 1977) and from the early embryo phase of the sea urchin, *Lytechinus pictus* (Chakrabarti et al., 1998). Chloral hydrate, probably through disassembly of microtubules, destabilizes the junction between the cilium and the basal body (Chakrabarti et al., 1998). It was also shown that chloral hydrate treatment disturbed the mitotic spindle in mouse oocytes by interfering with microtubules (Eichenlaub-Ritter & Betzendahl, 1995).

In this paper, we show that long-term incubation with chloral hydrate removes the cilia from MDCK cells and produces substantial changes in the organization of intracellular microtubules. Loss of the cilia eliminates the Ca^{2+} response of MDCK cells to changes in the apical flow rate, without affecting their response to direct mechanical stimulation of the apical membrane. When the deciliated cells are allowed to recover in normal cell culture medium for several days, the primary cilia grow back and the flow-induced Ca^{2+} response returns.

Materials and Methods

Cell Culture

Wild-type MDCK cells (passages 62–76 from the American Type Culture Collection, Rockville, MD) were grown to confluence on 25-mm diameter cover slips in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Gibco, Grand Island, NY) and 2 mM glutamine, but without riboflavin, antibiotics or phenol red, as previously described (Xia et al., 1998).

When 4 mM chloral hydrate was added to the culture medium, it was necessary to change the medium twice daily because the chloral hydrate slowly volatilized on exposure to air and its concentration fell.

Solutions

The perfusion solution had the following composition, in mM: [Na^+] 137, [K^+] 5.3, [Ca^{2+}] 1.8, [Mg^{2+}] 0.8, [Cl^-] 126.9, [SO_4^{2-}] 0.8, HEPES 14, glucose 5.6, probenecid 5, pH 7.4 (37°C, 300 mOsmol). Sources of chemicals were: Fluo-4-AM, anti-bovine α-tubulin mouse monoclonal antibody, ALEXA-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR), EGTA, probenecid, (Sigma, St. Louis, MO).

Microscopy and Perfusion

MDCK cell monolayers, grown on coverglasses, were viewed in a perfusion chamber at 37°C on the stage of an inverted microscope (Diaphot, Nikon, Melville, NY) equipped with differential interference contrast (DIC) combined with low light level fluorescence as described previously (Praetorius & Spring, 2001). Imaging was performed with a 100×/1.3 N.A. lens (Nikon) and an intensified CCD camera (ICCD-1001, Video Scope, Sterling, VA). The cellular fluorescence was sampled at a rate of 0.5 Hz, and measurements were initiated 50 seconds prior to the increases in perfusion rate from 2 to 8 μl sec^{-1}. Flow rates were calibrated by measurement of the efflux into a reservoir of known volume; a rate of 1 μl sec^{-1} corresponds to a linear velocity of 11 μm sec^{-1}, equivalent to a tubular flow rate of 7 nl min^{-1}.

An opening in the top coverglass of the perfusion chamber enabled the introduction of a micropipette that was mounted on a motorized micromanipulator. The micropipette allowed pressing the apical membrane of the MDCK cells. During these experiments the cells were under constant slow perfusion of 2 μl sec^{-1}.

Intracellular Ca^{2+} Measurements by Fluo-4

The cells were incubated for 15 minutes with the Ca^{2+}-sensitive probe Fluo-4-AM (5 μM) at 37°C, washed to remove excess probe. Then they were placed in the perfusion chamber and allowed at least a 20-minute de-esterification period. Fluo-4 fluorescence was measured in normal perfusion medium (i.e, free of chloral hydrate) as previously described (Praetorius & Spring, 2001). The fluorescence intensity was expressed relative to the baseline value, chosen as the mean of 5 intensity observations prior to the experimental manipulation. All solutions contained 5 mM probenecid to inhibit extrusion of the dye, and the experiments were carried out at 37°C, pH 7.4.

Immunofluorescence

MDCK cells, grown on glass coverslips for 4–8 days, were washed twice in phosphate-buffered saline (PBS) and fixed for 15 minutes in 2.5% formaldehyde at room temperature. Then the cells were washed twice and permeabilized with 0.3% Triton X-100 in PBS containing bovine serum (15 mg ml^{-1}) for 15 minutes. They were then incubated overnight with the primary antibody, anti-bovine α-tubulin mouse monoclonal (1 μg ml^{-1}) at 4°C. After thorough washing, the cells were incubated with Alexa-conjugated anti-mouse IgG (dilution 1/100), the secondary antibody, for 3 hours at room temperature. The cells were washed again and mounted in an anti-fade solution for observation with a confocal microscope (Odyssey, Noran Inst., Middleton, WI).

Statistics

All values are shown as the mean ± SEM. Statistical significance was determined using the nonparametric Mann–Whitney test, P values less than 0.05 were considered significant. The number of observations refers to the number of cells analyzed; typically six cells were studied in each preparation.

Results

Chloral Hydrate Treatment Results in the Loss of Primary Cilia

Exposure of MDCK cells to chloral hydrate (4 mM) for 20 hours changed the appearance of the primary cilia from the vertical, straight structures seen with anti-tubulin immunofluorescence under control conditions (Fig. 1*A*) to a curled and twisted geometry (Fig. 1*C*). In Fig. 1*A*, the focal plane is just above the apical membrane to visualize the primary cilia as small bright dots centrally located in the MDCK cells. Figure 1*B* shows the same cells as in Fig. 1*A* with the focal plane adjusted to that of the nucleus to allow the visualization of the cytoplasmic microtubule arrangement.

After 20 hours incubation with chloral hydrate, the primary cilia appear tangled and a few cells without cilia can be detected (Fig. 1*C*). The cytoplasmic microtubules have also changed their appearance from elongated structures to a more condensed and curled configuration (Fig. 1*D*). After 68 hours incubation with chloral hydrate, the primary cilia were undetectable (Fig. 1*E*), and the cytoplasmic microtubule organization was grossly distorted (Fig. 1*F*).

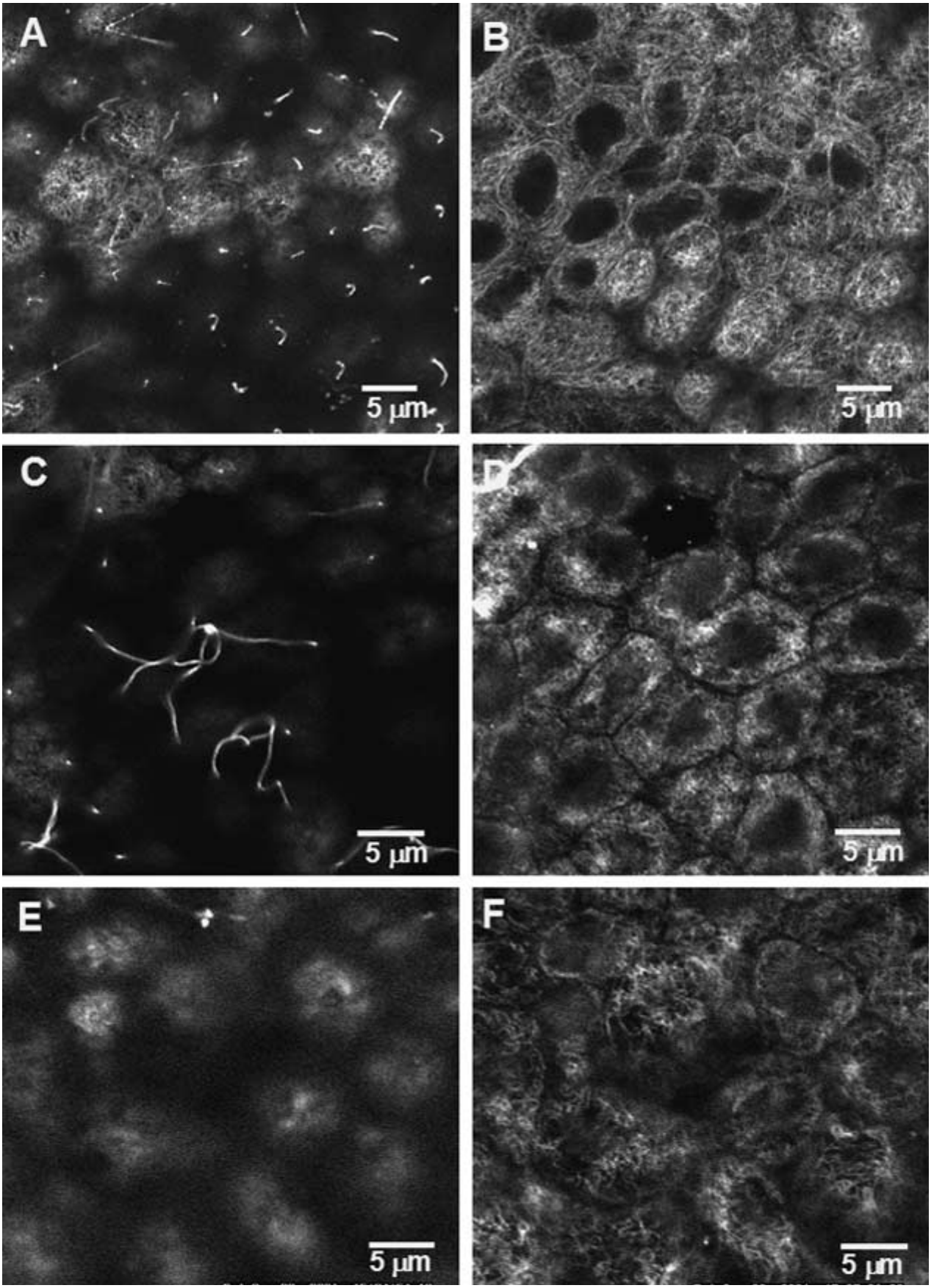

Fig. 1. Anti-tubulin immunofluorescence images of MDCK cells at two focal planes: *left*, at the level of the primary cilium; *right*, at the level of the cell nucleus. Panels *A* and *B* are images of control cells; *C* and *D* are images taken after 20 hours incubation with 4 mM chloral

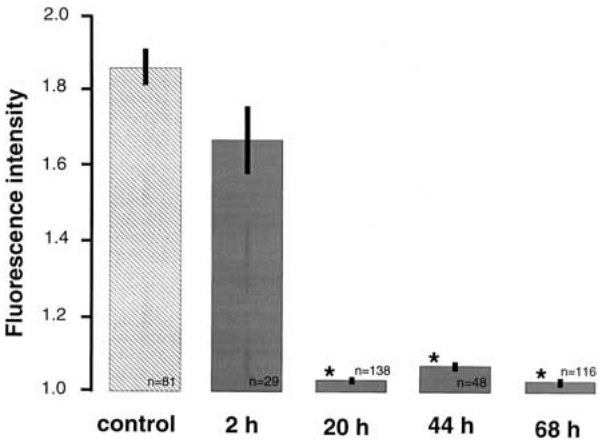

Fig. 2. Relative Fluo-4 fluorescence, a measure of $[Ca^{2+}]$, in response to an increase in perfusion rate from 2 to 8 μl sec^{-1}. Dark bars show the response after treatment with chloral hydrate for the indicated time periods. Asterisks indicate a significant difference from the control.

Loss of the Ca^{2+} Response to Flow

Figure 2 shows that control MDCK cells respond to an increase in the flow rate of perfusate with an increase in the relative Fluo-4 fluorescence, and hence $[Ca^{2+}]_i$, by 1.85 ± 0.05 (n = 81). Preincubation with chloral hydrate (4 mM) for 2 hours slightly reduced the flow-induced response to 1.66 ± 0.09 (n = 29). However, longer incubation caused a progressive decrease in the response to changes in apical flow rate with complete loss after 20 hours of exposure. The Fluo-4 fluorescence was 1.03 ± 0.01 (n = 138) after 20 hours, 1.08 ± 0.01 (n = 48) after 44 hours and 1.03 ± 0.001 (n = 116) after 68 hours of exposure to chloral hydrate.

Recovery of the Primary Cilia

When the MDCK cells were allowed to recover in normal cell culture medium after chloral hydrate treatment, they slowly regained a primary cilium (Fig. 3). Figure 3*A* shows MDCK cells that have been incubated 68 hours with chloral hydrate, and allowed to recover for 24 hours in normal medium. At this point, cilia were not observed, but the intracellular microtubules have returned to nearly normal appearance (Fig. 3*B*). Even after 72 hours of recovery, no cilia could be detected although the intracellular microtubules appeared indistinguishable from control. A nodular condensation on the apical membrane started to appear after 96 hours recovery and a few short cilia were visible (Fig. 3*C*). Only after 120 hours of recovery did most cells have cilia of about 3–4 μm in length (Fig. 3*E*).

Recovery of the Ca^{2+} Response to Flow

Figure 4 shows that the Ca^{2+} response to increasing flow rates was completely absent until 96 hours after recovery when a submaximal Ca^{2+} response of 1.32 ± 0.04 (n = 102) was seen. This value was the mean of a large number of observations, in which only a few cells responded to the flow change, while the majority did not. (The very short cilia were not consistently visualized with DIC and thus we could not determine whether the responsive cells had a more developed cilium than the non-responders. Clear visualization of the cilia and determination of their length required fixation of the cells and staining for immunofluorescence). After 120 hours of recovery, the Ca^{2+} response to flow was indistinguishable from the control, 1.79 ± 0.07 (n = 62).

Response to Pressing the Cell Membrane with a Micropipette

Since the flow-induced Ca^{2+} response is known to be dependent on CICR involving the mobilization of intracellular Ca^{2+} stores (Praetorius & Spring, 2001), the failure to sense flow rate changes caused by long-term incubation with chloral hydrate could be the result of a breakdown in the machinery for CICR and be unrelated to the expression of the primary cilium.

MDCK cells respond to contacting the apical membrane by a micropipette with a large increase in $[Ca^{2+}]_i$ that spreads by the diffusion of IP_3 through gap junctions from the disturbed cell to its neighbors (Praetorius & Spring, 2001). The amplitude of this mechanically-induced Ca^{2+} response and its spread to neighboring cells was virtually unchanged by incubation with chloral hydrate (Fig. 5). Under control conditions, contacting the membrane induced an increase in the relative Fluo-4 fluorescence of 3.85 ± 0.24 (n = 10), and the Ca^{2+} signal from the disturbed cell spread to 88 out of 91 adjacent cells. Figure 5 shows that incubation with chloral hydrate did not significantly affect the magnitude of the increase in relative Fluo-4 fluorescence upon pressing the apical membrane. Chloral hydrate treatment had, at most, only a minor effect on the spreading of the Ca^{2+} signal to adjacent cells. After 44 hours of incubation in chloral hydrate, pressing the apical membrane resulted in spreading of the Ca^{2+} signal to 45 of 56 adjacent cells. After 68 hours in chloral hydrate, the Ca^{2+} wave spread to 49 of 65 adjacent cells. When the treated cells were allowed to recover in normal cell culture medium for 24 hours, the Ca^{2+} signal spread to 49 of 56 neighboring cells. After a recovery period of 120 hours, the signal spread to 91 of 93 adjacent cells.

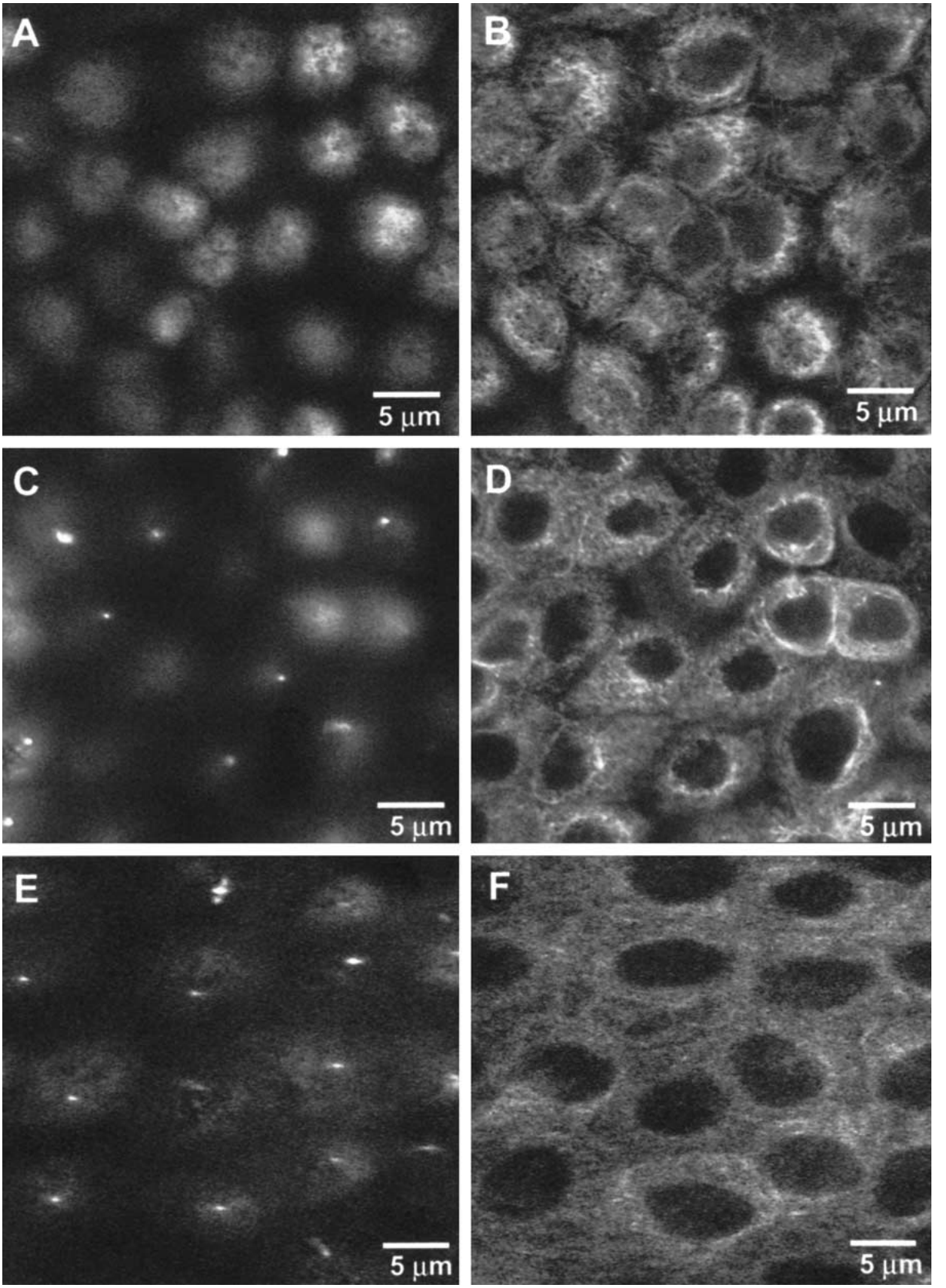

Fig. 3. Anti-tubulin immunofluorescence images of MDCK cells that had been exposed to chloral hydrate for 68 hours and then returned to normal cell culture medium. Images on the left were taken with the focus at the level of the apical surface; images on the right, with the focus at the level of the nucleus. Panels *A* and *B* show the cells after 24 hours in normal medium; *C* and *D*, after 96 hours; *E* and *F*, after 120 hours.

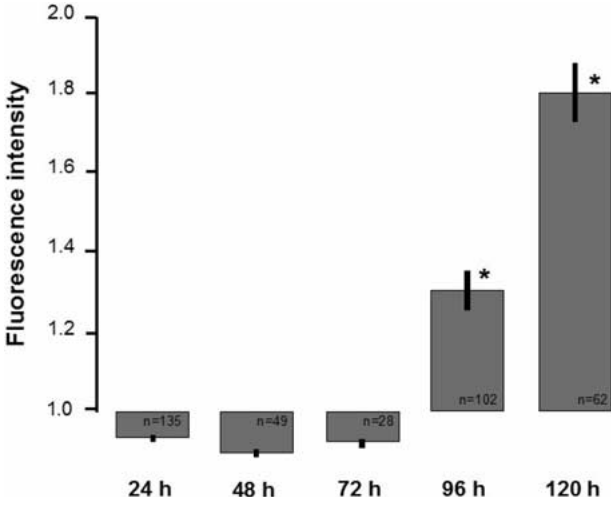

Fig. 4. Relative Fluo-4 fluorescence, a measure of $[Ca^{2+}]$, in response to an increase in perfusion rate from 2 to 8 μl sec^{-1}. Cells were treated with chloral hydrate for 68 hours and then returned to normal cell culture medium for the indicated time periods. Asterisks indicate a significant difference from 1.0.

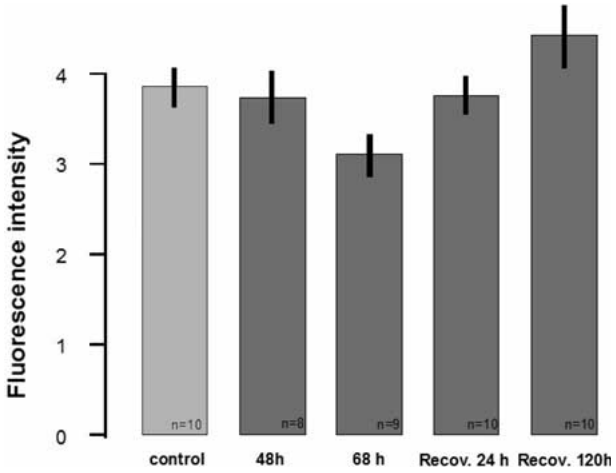

Fig. 5. Relative Fluo-4 fluorescence is shown for the response to contacting the apical membrane with a micropipette. Dark bars indicate the response after exposure to chloral hydrate for 48 or 68 hours or recovery in normal medium for 24 or 120 hours following 68 hours in chloral hydrate.

Time Course of Ca^{2+} Response Stimulated by Flow

The data on the increase in intracellular Ca^{2+} in Figs. 2 and 4 and in our previous study (Praetorius & Spring, 2001) utilized continuous bending of the cilium by maintenance of the suction of the micropipette or by continuous flow of perfusate. In the experiments shown in Fig. 6, the period of increased flow rate of perfusate was varied to determine the minimum duration of cilia bending required to initiate a Ca^{2+} response. The rate of perfusion was suddenly increased from 2 to 8 μl sec^{-1} for the time period indicated on the abscissa and the peak magnitude of the subsequent fluorescence change is indicated on the ordinate. A bending period of 3 seconds was required to produce a response of control magnitude, but a significant response could be detected after only two seconds of flow.

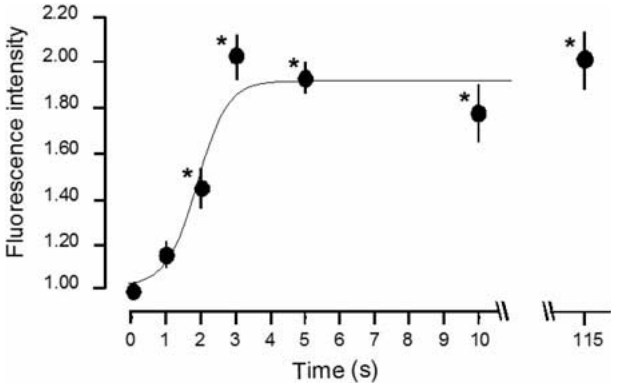

Fig. 6. Flow-induced Ca^{2+} response of Fluo-4-loaded MDCK cells when the perfusion rate was increased from 2 to 8 μl sec^{-1} for the times indicated on the abscissa. Asterisks indicate values that differ significantly from the value at 0 sec. Error bars indicate the SEM. The total number of tested cells: $n = 6$, 0 sec; $n = 34$, 1 sec; $n = 26$, 2 sec; $n = 37$, 3 sec; $n = 35$, 5 sec; $n = 16$, 10 sec; $n = 22$, continuous flow.

Discussion

The Cilium is the Sole Flow Sensor

The correspondence between the expression of a primary cilium by MDCK cells and the cells' ability to sense fluid flow (i.e., increase $[Ca^{2+}]_i$ in response to flow) leads us to the conclusion that the cilium is the sole flow sensor in this epithelium. Although our previous studies (Praetorius & Spring, 2001) were consistent with this hypothesis, the evidence was not conclusive because of the lack of a method for removal of the cilia from mature MDCK cells. The possibility that MDCK cells could sense flow by some other mechanism, e.g., shear stress, could not be excluded, although it seemed unlikely. The results of the present study directly address that question and show that a primary cilium of adequate length is required for flow sensing. Flow sensing was completely abolished in deciliated cells returned to normal medium for 72 hours, a time when cilia were still not detectable by immunofluorescence. The flow response only fully recovered after 120 hours, at which time all cells exhibited cilia. During 96 to 120 hours after return to normal medium, the cilia increased in length and the response to flow became widespread and similar to

that of control cells. Clearly, the growth of the cilium to a critical length or the expression of the mechanosensitive proteins in the cilium must occur during this time period.

Effects of Chloral Hydrate

It has previously been determined that chloral hydrate treatment not only weakens the attachment of the primary cilium to the cell at the basal body, but also causes substantial alterations in microtubules and associated processes, such as formation of the mitotic spindle (Eichenlaub-Ritter & Betzendahl, 1995). Significant disturbance of microtubules was evident in MDCK cells after as little as 20 hours of exposure to chloral hydrate (Fig. 1*D*). The effects of chloral hydrate on microtubules in the primary cilium were also readily apparent from the changes in the cilium's shape (Fig. 1*C*). Although the prolonged incubation time required to remove the cilia and the broad range of effects of the drug were undesirable, our attempts with drugs or methods used for isolating cilia from lung (Weaver & Hard, 1985) were unsuccessful. It is unlikely that the effects of chloral hydrate on intracellular microtubule organization were causally related to the loss of the primary cilia since Jensen et al. (1987) showed that agents causing microtubule disruption in PtK_1 cells did not affect their cilia. The adverse effects of chloral hydrate on MDCK cell microtubules disappeared at least two days before the flow response returned. Thus, these two phenomena are temporally disconnected and are probably unrelated. It was previously shown that the Ca^{2+} increase from contacting the membrane has a profile other than that produced by bending the cilium and does not require extracellular Ca^{2+} (Praetorius and Spring, 2001). However, the present touch-induced Ca^{2+} responses of MDCK cells suggest that chloral hydrate did not alter the Ca^{2+} mobilization processes involved in CICR or in spread of the Ca^{2+} signal from cell to cell.

At present, chloral hydrate is the only effective tool for removal of MDCK cell cilia even though its actions are not solely restricted to severing the attachment of the cilium to the basal body.

Only a Small Ca^{2+} Influx Is Required

On the basis of our previous investigations and those of the present study, we conclude that Ca^{2+} must first enter the cell through mechanically sensitive channels in the primary cilium. It is instructive to estimate the magnitude of the requisite Ca^{2+} influx as well as the number of channels needed to achieve such an influx.

A typical MDCK cell primary cilium has a length of 8 μm and a radius of 0.1 μm, resulting in a calculated volume of 0.25×10^{-15} l (0.25 fl). This should be compared to the measured MDCK principal cell volume of 800 fl (Kovbasnjuk et al., 1995). If the free Ca^{2+} concentration, $[Ca^{2+}]_{free}$, in the cilium is the same as that in the cell, ~150 nM (Woda et al., 2000, 2001), there are about 3.8×10^{-23} moles of free Ca^{2+} in the cilium, or only one molecule. In the absence of a Ca^{2+}-buffering system in the primary cilium, an influx of 4 Ca^{2+} ions would be sufficient to increase $[Ca^{2+}]_{free}$, to 1 μM. However, Ca^{2+} is heavily buffered in all cells, limiting its intracellular diffusion to distances of 3–6 μm, and resulting in $[Ca^{2+}]_{free}$ of about 10^{-4} of the $[Ca^{2+}]_{Total}$ (Höfer et al., 2001). If Ca^{2+} in the cilium were similarly buffered, the influx required to increase the free Ca^{2+} to 1 μM would be far larger or about 60,000 Ca^{2+} ions (1×10^{-19} moles). Our results show that three seconds of stimulation by flow were required to produce CICR in MDCK cells, so the calculated Ca^{2+} influx would be about 20,000 ions sec^{-1} (0.33×10^{-19} moles sec^{-1}). Such a Ca^{2+} influx is potentially measurable, as Ca^{2+} influxes have been measured in dendritic spines with a volume of 0.05–0.1 fl (Sabatini & Svoboda, 2000).

Estimation of the Number of Channels

How many mechanically-sensitive channels would be needed to achieve a Ca^{2+} influx of 20,000 ions sec^{-1}? The polycystins are the most likely candidate molecules for the mechanically-sensitive channel in the primary cilium, as they share many of the characteristics of the Ca^{2+} entry pathway in MDCK cells. Two proteins, polycystin-1 and polycystin-2, associate to form a mechanically-sensitive cation channel that is somewhat selective for Ca^{2+} (Chen et al., 1999; Vandorpe et al., 2001). The inhibitor sensitivity of this polycystin complex is remarkably similar to that of the MDCK cell flow sensor (Praetorius & Spring, 2001). Both are completely inhibited by Gd^{3+} and partially blocked by amiloride (unpublished observation by Praetorius and Spring) but insensitive to the traditional blockers of Ca^{2+} channels. Homologs of polycystin 1 and 2 are known to be located in the sensory cilium of *C. elegans* (Barr et al., 2001), however, recently it was revealed that Polycystin 2 in cultured mouse and human kidney cells is localized to the primary cilium (Pazour et al., 2002).

Polycystin channels have a conductance of ~120 pS (Chen et al., 1999; Vandorpe et al., 2001). The calculated total cationic current through a polycystin channel driven by a transmembrane potential difference of −23 mV, would be 2.8 pA or 2.9×10^{-17} moles sec^{-1}. The fraction of the current constituted by Ca^{2+} ions can be calculated from the extracellular Ca^{2+} concentration (1.8 mM), the channel open probability (0.1) and the channel's relative Ca^{2+}/Na^{+} conductance (4/1) as 1.4×10^{-19} moles sec^{-1} or 84,000 ions sec^{-1}. This flux is about four times larger than

the estimated influx for CICR, and, thus, a single polycystin channel in each primary cilium would be sufficient for flow sensing. If only one channel were, indeed, responsible for sensing fluid flow, restriction of its location to one side of the cilium could also confer directional sensitivity to the response.

In summary, our results strongly support the conclusion that the primary cilium is the only mechanism for flow sensing by MDCK cells. We estimate that the requisite Ca^{2+} influx could be accomplished by a single polycystin complex in the primary cilium.

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